

Protocol Sheet

Roche® LightCycler® 480

real-time PCR run setup instructions for miScript® miRNA PCR Arrays

Important points before starting

- Please read the handbook supplied with the miScript miRNA PCR Array, paying careful attention to the “Safety Information” and “Important Notes” sections, before beginning this procedure.
- Make sure the real-time PCR instrument is working properly. Refer to the manufacturer’s Installation and Maintenance manual if needed.
- Make sure the correct thermal block cycler (384-well block format for G plate or 96-well for F plate) is in place.
- Turn on the LightCycler 480 instrument; the main switch is located at the back of the instrument. Turn on the computer linked to the instrument and log on to Windows® XP.

Procedure

Creation of PCR protocol template

1. Start LightCycler 480 software 1.5.0 SP3 (version 1.5.0.39).
2. Enter “Username” and “Password” when the “Login” dialog box appears and click the  button to proceed with the login. The “Overview” window will appear.
3. Wait for the initiation of the instrument to finish and the two status LEDs on the front of the instrument to become stable. The left LED should become a steady green while the right LED should be a steady orange.
4. In the “Overview” window (Figure 1), click the “New Experiment” button on the right-hand side of the window to launch the “Run” module.



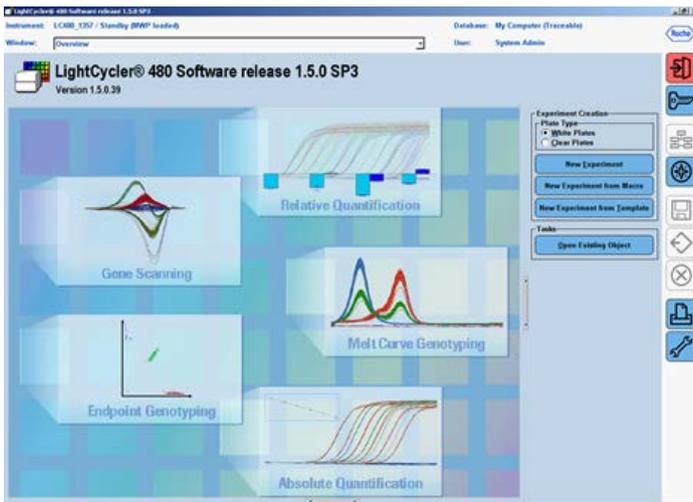


Figure 1. Overview window.

5. In the “Setup” area at the top of the “Run Protocol” tab (Figure 2), specify the following setup parameters:

- Detection Format: SYBR® Green I
- Block Type: should show the correct block format (384 or 96)
- Plate ID: (Optional)
- Reaction Volume: 10 μ L for G plate or 25 μ L for F plate

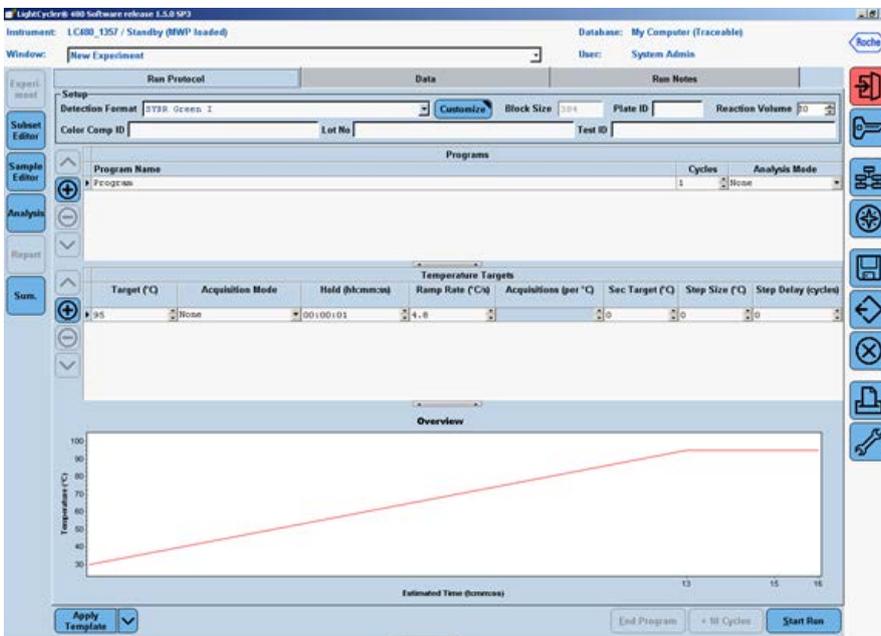


Figure 2. Initial run protocol window.

- In the "Programs" section, click the  button twice to add two more program lines.
- Click to change the "Program Name" of the first program to "Heat Activation". Leave the number of Cycles at "1" and leave the Analysis Mode as "None". In the "Program Temperature Targets" section, enter the values for the following parameters (see Figure 3):

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:15:00	4.8*		0	0	0

* This is the default ramp rate for 384-well block. The default ramp rate for 96-well block is 4.4 °C/s.

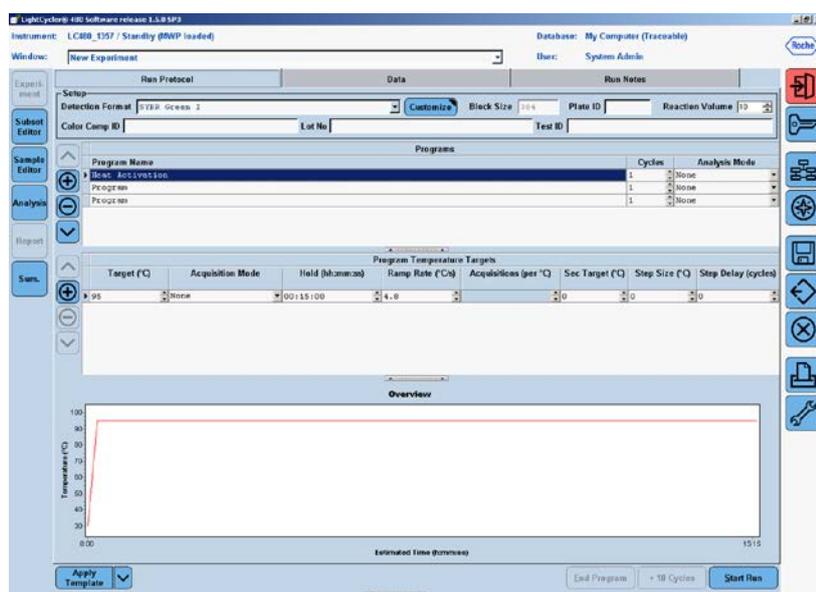


Figure 3. Setup for the first program — Heat Activation.

- Click to change the "Program Name" of the 2nd program to "PCR Cycling". Change the number of Cycles to "45"[†] and change the Analysis Mode to "Quantification". In the "Temperature Targets" section, click the  button twice to add two more temperature target lines. Enter the values for the following parameters (Figure 4):

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
94	None	00:00:15	1.0		0	0	0
55	None	00:00:30	1.0		0	0	0
70	Single	00:00:30	1.0		0	0	0

[†] The PCR is run for extra five cycles (i.e. 45 cycles) rather than the typical 40 cycles indicated in the *miScript miRNA PCR Array Handbook* as this will allow the use of the Second Derivative Maximum method available with the LightCycler 480 software for data analysis. See Data Analysis section for further details.

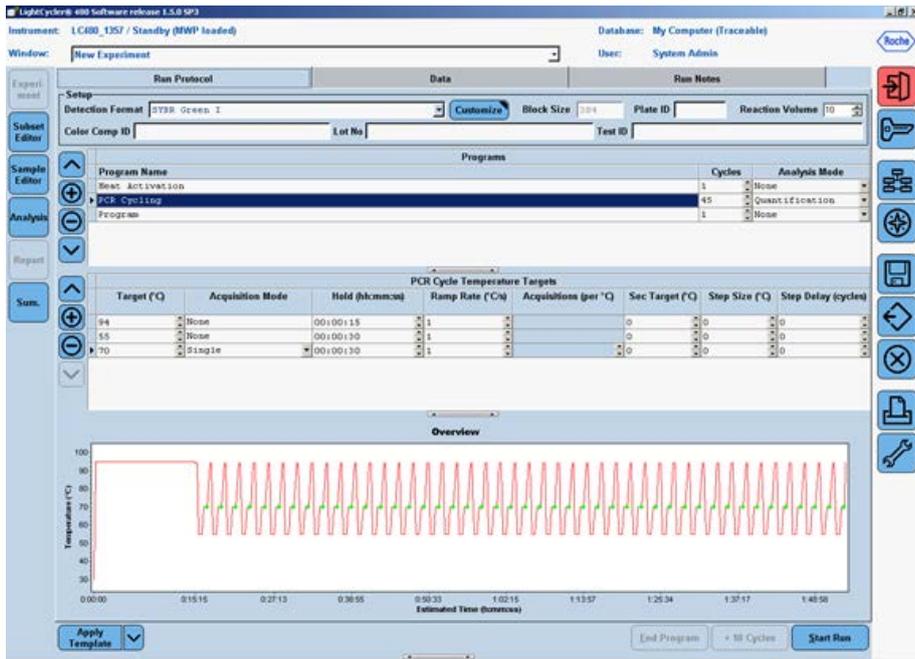


Figure 4. Setup of the second program — PCR Cycling.

- Click to change the “Program Name” of the 3rd program to “Melt Curve”. Leave Cycles at “1” and change Analysis Mode to “Melting Curves”. In the “Temperature Targets” section, click the  button once to add one more temperature target line. Enter the values for the following parameters (Figure 5):

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
60	None	00:00:15	2.5 [‡]				
95	Continuous		0.03	20			

[‡] This is the default ramp rate for the 384-well block. The default ramp rate for the 96-well block is 2.2°C/s.

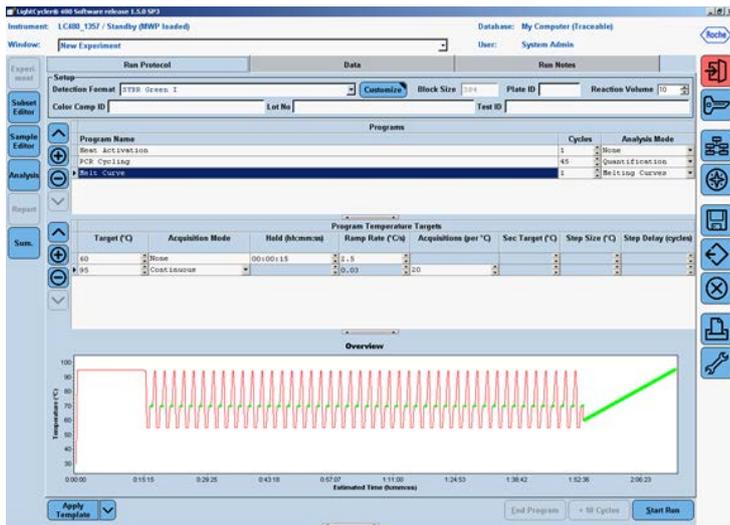


Figure 5. Setup of the third program — Melt Curve.

10. Click the down arrow button (▼) right next to the “Apply Template button on the lower left corner of the screen; select the Save As Template button (Figure 6) to save the run template under the filename “miScript_miRNA_PCR_Array_384_LC” if using the 384-well format or “miScript_miRNA_PCR_Array_96_LC” for if using the 96-well format.

Note: Alternatively, download the PCR protocol template file (miScript_miRNA_Template_LC480_96 or miScript_miRNA_Template_LC480_384) from the QIAGEN website, www.qiagen.com.

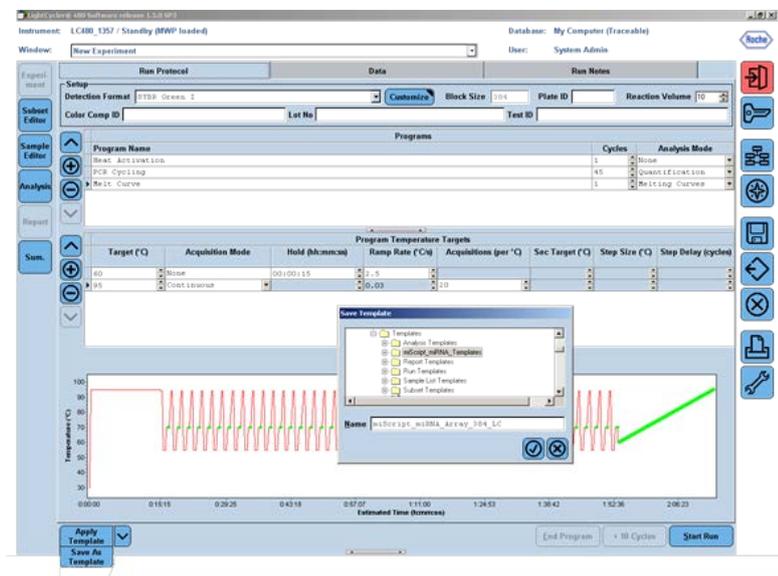


Figure 6. Saving the “Run Template” file.

Real-time PCR detection

To load the PCR array plate and start the run:

11. Prepare the PCR array following the *miScript miRNA PCR Array Handbook*.
12. Make sure the correct thermal block (384-well block format for G plate or 96-well for F plate) is in place.
13. If the instrument is not on, turn it on using the main switch at the back of the instrument. Turn on the computer linked to the instrument and log on to Windows® XP.
14. Start LightCycler 480 software (version 1.5.0.39).
15. Enter "Username" and "Password" when the "Login" dialog box pops up and click the  button to proceed with the login. The "Overview" window will appear.
16. Wait for the two status LEDs on the front of the instrument to become stable. The left LED should become a steady green while the right LED should be a steady orange.
17. Press the ←→ open/close button on the front of the instrument (located next to the instrument status LEDs). The plate loading arm will slide out of the right side of the instrument. Place the PCR array plate into the frame of the loading arm with the flat edge of the array closest to the instrument and the edge with a beveled corner pointing away from the instrument.
 - Press the ←→ open/close button again to retract the loading arm. Wait for both of the status LEDs to turn steady green.
 - In the "Overview" window, click the "New Experiment" button on the right-hand side of the screen.
 - In the "Run Protocol" tab, click the "Apply Template" button on the lower left corner of the screen. Select and double click to open the appropriate previously saved "miScript_miRNA_PCR_Array_384_LC" or "miScript_miRNA_PCR_Array_96_LC" run template file.
 - Press the "Start Run" button on the lower right corner of the screen to initiate the run. When prompted, save the file under a new filename after which the run will begin.

Data analysis

18. In the "Navigator" window (Figure 7), find and open the run file to be analyzed.

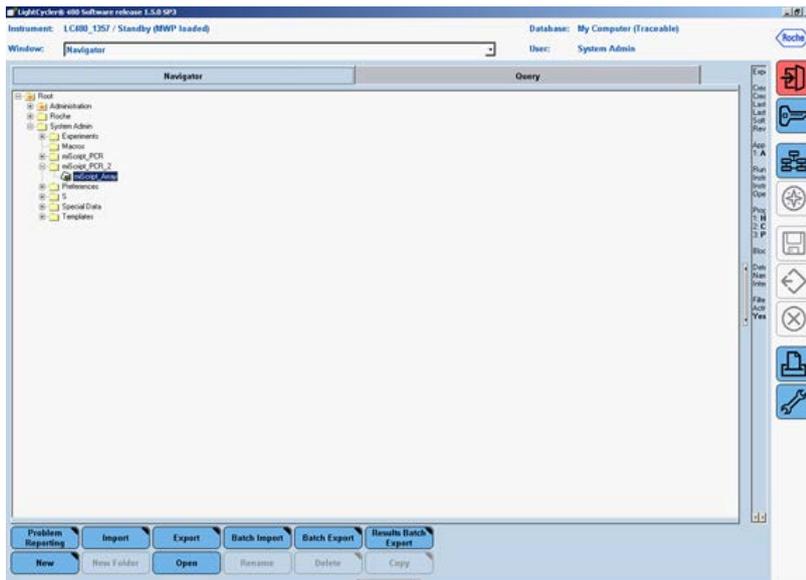


Figure 7. Opening a completed run file.

19. For data analysis using the LightCycler 480, we highly recommend the use of the **Second Derivative Maximum** analysis method available in this software. This is an algorithm based on the kinetics of a PCR reaction. This method identifies the crossing point (Cp) of a PCR reaction as the point where the reaction's fluorescence reaches the maximum of the second derivative of the amplification curve, which corresponds to the point where the acceleration of the fluorescence signal is at its maximum. Hence, this crossing point should always be located in the middle of the log-linear portion of the PCR amplification plot. The advantage of this analysis method is that it requires little user input and produces consistent results.
20. **Obtaining the Crossing Point (Cp) Cycle values:** Once the selected run file is open, click the "Analysis" button on the left-hand side of the window. In the "Create new analysis" list, double-click to select "Abs Quant/2nd Derivative Max". In the "Create new analysis" window that opens, make sure the "Analysis Type" is "Abs Quant/2nd Derivative Max" (Figure 8). Click  to confirm the selection.

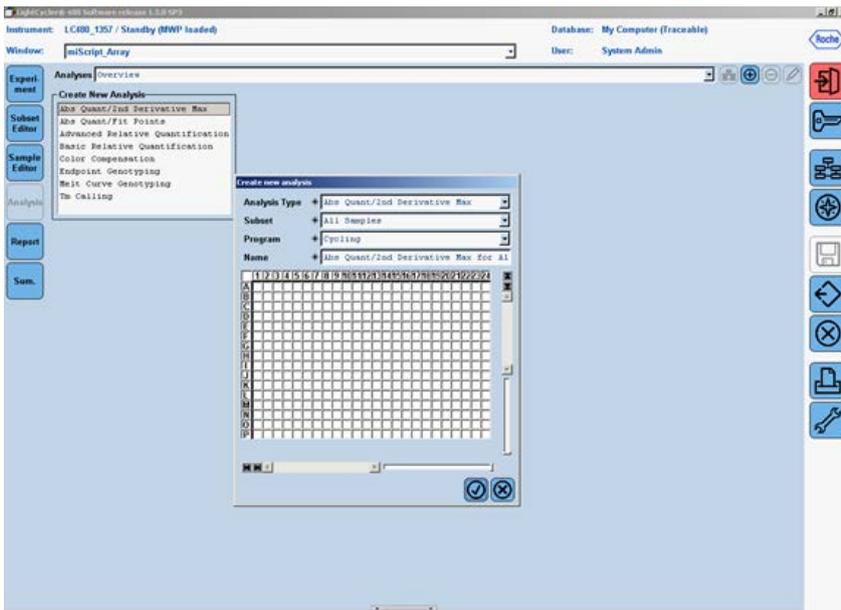


Figure 8. Setting up the analysis for the Second Derivative Maximum.

21. The “Analysis Window” with the amplification curves will appear (Figure 9). To proceed, click the down arrow button () on the lower right corner of the screen to set the analysis to “High Confidence.” Next click the “Calculate” button to generate Cp values. The calculation may take a few minutes.

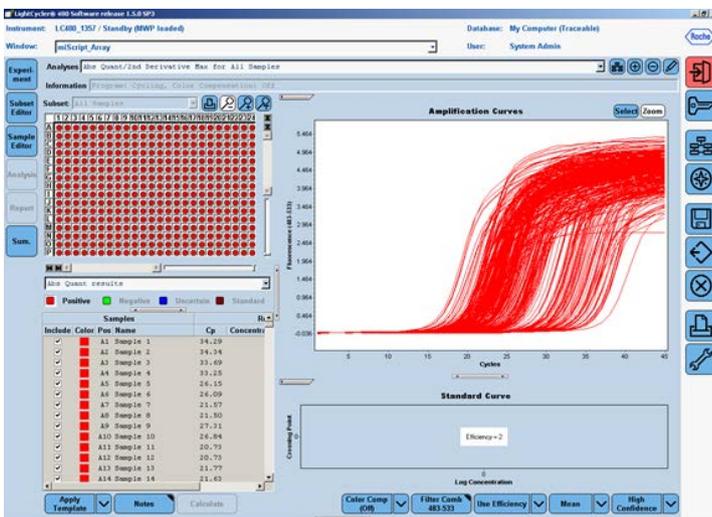


Figure 9. Obtaining the Crossing Point (Cp) Cycle values at the Second Derivative Maximum.

22. Once the Cp values are generated, move the mouse over to the Samples window and click to highlight the Samples result table. Right click the “Export” button to export the data. A “Save table data” dialog box will appear (Figure 10). Enter a filename to save the data as a text file. Then click the  button on the right panel to save the Second Derivative Maximum Analysis and the Cp values as part of the run file.

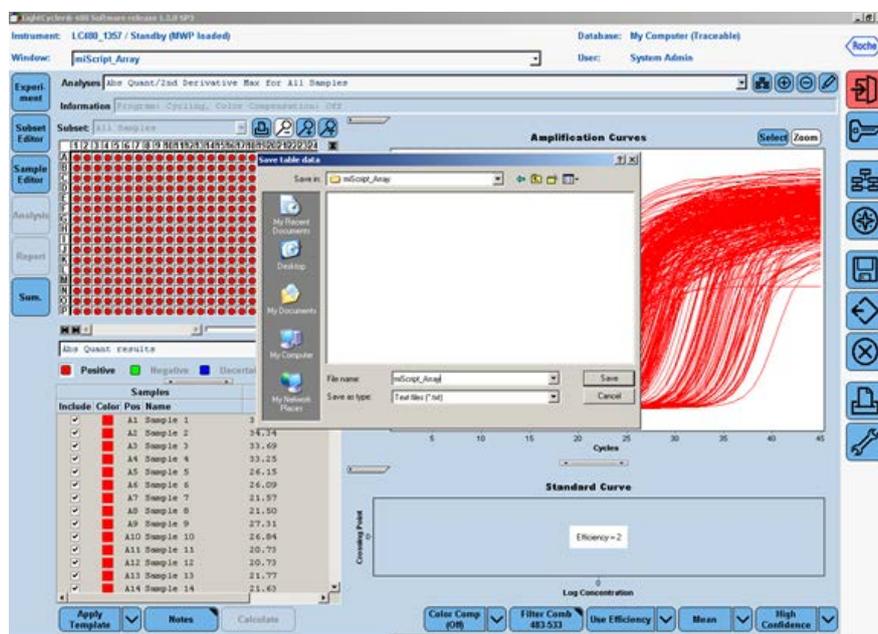


Figure 10. Exporting the Cp values.

23. To proceed with the melt curve analysis, click the  button located on the right of the “Analyses” line at the top of the window. The “Create New Analysis” dialog box will open (Figure 11). Select the “T_m calling” for the “Analysis Type.” Then click  to confirm the selection.
24. The “Analysis” window with the raw melt curves will appear (Figure 12). To proceed, click the down arrow button () on the lower right corner of the screen to set the analysis to “SYBR Green I Format”. Next click the “Calculate” button. The calculation may take several minutes. Once the melting peaks and T_m values appear, check the specificity of each assay by ensuring the presence of a single melt peak in each well. Then click the  button on the right panel to save the melt curve analysis as part of the run file.

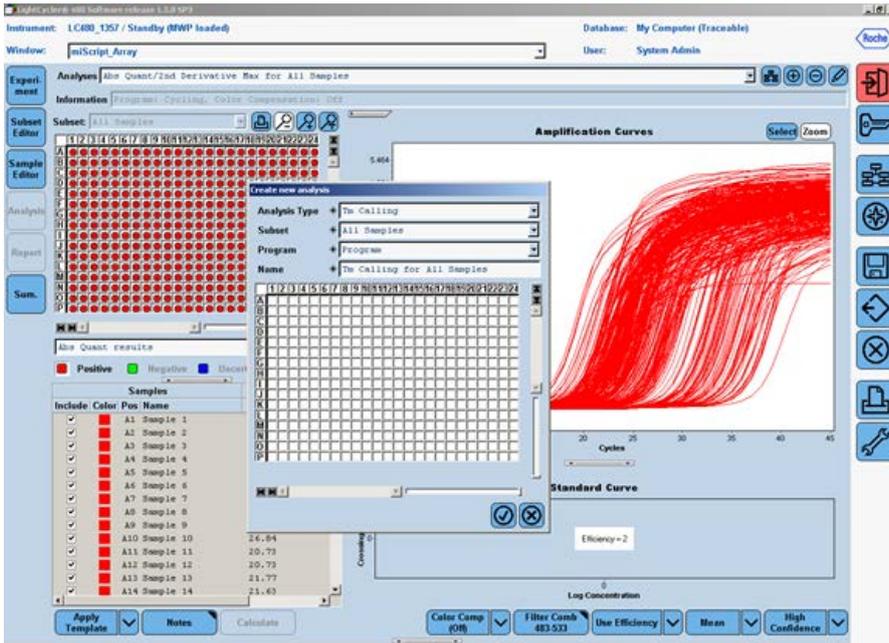


Figure 11. Setting up the melt curve analysis.

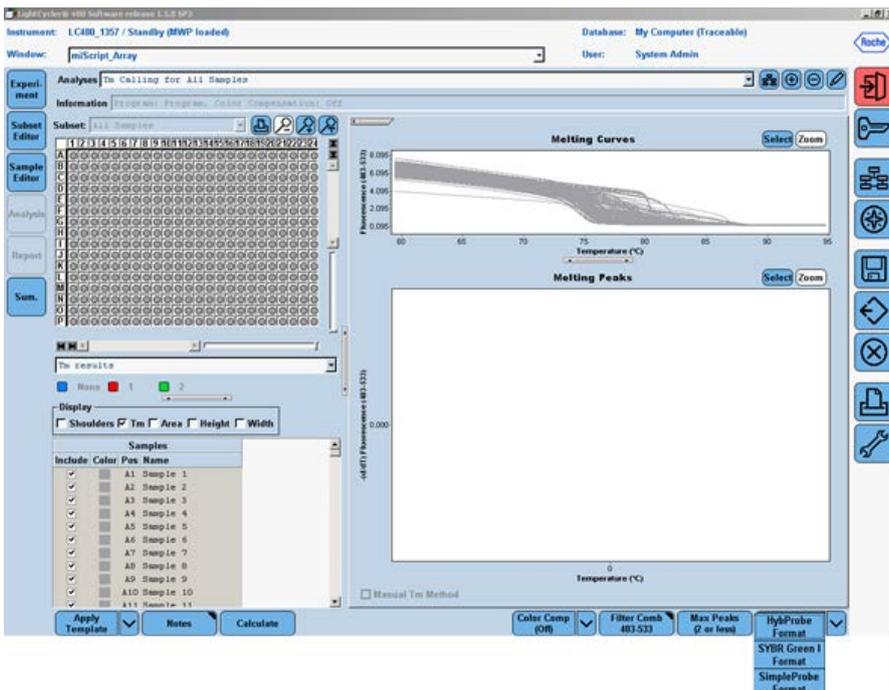


Figure 12. Generating the melt curve analysis.

25. To save the melt peak values: Move the mouse over to the "Samples" window and click to highlight the result table. Right-click the "Export" button to export the data. A "Save table data" dialog box will appear (Figure 13). Enter a filename to save the data as a text file.

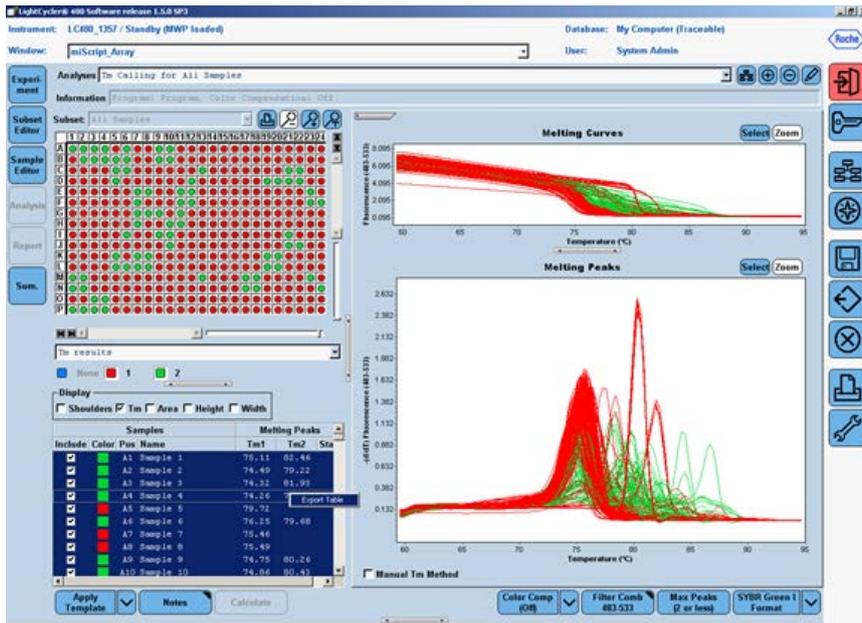


Figure 13. Exporting the melt curve data.

The miScript miRNA PCR Array is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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